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Force Analysis of Bacterial Transmission from Contact Lens Cases to Corneas, with the Contact Lens as the Intermediary

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Purpose. To determine the probability of transmission of a *Staphylococcus aureus* strain from a contact lens case, to the contact lens (CL) surfaces, to the cornea, on the basis of bacterial adhesion forces measured by using atomic force microscopy (AFM).

Methods. Adhesion forces between *S. aureus* strain 835 probes with rigid and soft CLs, storage cases, and porcine corneas were measured with AFM and used to calculate Weibull distributions, from which the transmission probability from one surface to another was derived. Bacterial transmission probabilities from force analyses were compared with experimentally obtained transmission data.

Results. After bond-strengthening, *S. aureus* adhered to the surface of a lens case with a median force of 10.8 nN. Adhesion forces were different on the soft and rigid CLs (7.7 and 13.6 nN, respectively). Adhesion forces on porcine corneas amounted to 11.8 nN. Data variations were used to calculate the Weibull distribution, from which the probability of transmission from the lens case to a CL and from the CL to the cornea can be directly read. Final transmission probabilities from lens case to the cornea were slightly higher for the rigid (24%) than for the soft (19%) CL. Bacterial transmission determined experimentally increased with increasing contact times, but were within the range of the probabilities derived from Weibull analyses.

Conclusions. Probabilities of bacterial transmission from contaminated lens cases to corneas can be derived from Weibull analyses of measured forces of adhesion to the surfaces involved. (*Invest Ophthalmol Vis Sci.* 2011;52:2565–2570) DOI:10.1167/iovs.10-6392

Wearing contact lenses (CLs) is one of the main risk factors for microbial keratitis, besides ocular trauma, ocular surgery, and ocular surface disease, and can lead to impairment of vision.[1–3] Moreover, the wearing of CLs may impair the immune response of the cornea by distorting its epithelial barrier function, contributing to the development of keratitis.[4] The incidence of microbial keratitis is only 0.02% to 0.5%,[5,6] but considering that 80 to 90 million people worldwide are wearing CLs for the correction of refractive errors,[7] it poses a major health threat. The most popular CLs are soft, silicone-hydrogel (S-H), and rigid gas-permeable (RGP) lenses because of their high oxygen permeability.[8] The annual incidence of microbial keratitis in soft CL wearers is 5.5 per 10,000, whereas in RGP wearers it is 1.2 per 10,000.[9] Different microbial strains and species have been isolated from microbial keratitis, from which approximately two thirds are Gram-negative species, notably *Pseudomonas aeruginosa*, but also *Serratia* species, whereas one third involve Gram-positive cocci, including *Staphylococcus aureus* and *Staphylococcus epidermidis* strains.[10]

Bacterial adhesion to CLs is one of the crucial steps in microbial keratitis. Bacterial adhesion is initially reversible, but becomes irreversible within several tens of seconds due to interfacial rearrangements between the bacterium and the substratum surface, a process generally referred to as bond-strengthening. When a contaminated CL is placed on the eye, bacteria can detach and adhere to the cornea.[11,12] CLs themselves can become contaminated during insertion, but also bacterial detachment from contaminated lens cases followed by subsequent adhesion to a CL can lead to bacterial contamination of a CL and therefore the cornea.[13] The development of microbial keratitis is thus initiated by transmission of organisms from lens case to CL to cornea, although actual occurrence of keratitis also depends on the virulence of the bacteria involved and the inflammatory and immune responses from the host.[14] Whether transmission from one surface to another occurs depends on the balance between the force of bacterial adhesion to one surface and the detachment force exerted by the opposing surface. A strong adhesion force implies that detachment and subsequent transmission to another surface is difficult, depending on the forces exerted by the receiving surface.

The forces of bacterial adhesion to surfaces involved in the development of microbial keratitis have never been measured and compared for the possibility that they will be transmitted to the cornea, although the force of bacterial adhesion to surfaces can be directly measured by atomic force microscopy (AFM).[15,16] In bacterial adhesion force measurements with AFM, a bacterium is attached to a cantilever with a known spring constant and brought into contact with a substratum surface. On retraction of the bacterium away from the substratum, the bending of the cantilever due to the adhesion force between the bacterium and substratum is measured and used to calculate the exact force. Statistically significant conclusions from adhesion forces obtained by AFM are difficult to draw, unfortunately, because of a large data spread arising from differences between individual bacteria or heterogeneities on a substratum or bacterial cell surface. Weibull analysis, common in macroscopic bond-strength analyses,[17] takes advantage of

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this spread to derive a Weibull distribution, yielding the probability that occurrence a force level will be reached and the dependability of the data set. Recently, it has been shown that Weibull analysis is applicable to nanoscopic bacterial adhesion forces obtained with AFM as a macroscopic bond-strength analysis.[18]

The purpose of this study was to use AFM to determine the Weibull distributions for bacterial adhesion forces of a *S. aureus* strain on CL cases, both soft and rigid CLs, and corneal surface. Subsequently, the Weibull distributions are used to calculate the transmission probabilities between case and CL, CL and cornea, and case to cornea via the CL. The probabilities of bacterial transmission determined by force analyses were compared with experimentally obtained transmission data.

Materials and Methods

Bacterial Cultures

S. aureus 835, a hydrophilic staphylococcal strain, was obtained from the Department of Medical Microbiology, University Medical Center Groningen. From a frozen stock, bacteria were precultured for 24 hours at 37°C in 10 mL tryptone soya broth (TSB; Oxoid, Basingstoke, UK). The preculture was used to inoculate a second culture for 18 hours at 37°C. *S. aureus* 835 was harvested by centrifugation for 5 minutes at 5000*g*. The bacteria were washed twice and resuspended in demineralized water. For transmission experiments, the bacteria were suspended to a density of 3×10^4 cells/mL in 0.9% NaCl supplemented with 2% (wt/vol) TSB to stimulate metabolic activity and adhesion but prevent growth.

Contact Lenses and Storage Cases

The soft CL included in this study was made of lotrafilcon A, containing 24% water (Focus-Night-and-Day; Ciba Vision, Duluth, GA) and belonging to FDA class I. The RGP CL used was made of enflufocon A (Boston-ES, fluorsilicone acrylate; Polymer Technology Inc., Clifton, NJ). A standard polypropylene screw-top contact lens case was used.

The RGP lenses and the lens cases were used several times and cleaned between the different measurements by sonication in an ultrasonic bath (Transsonic TP 690; Elma, Singen, Germany) for 5 minutes in 0.9% NaCl, thoroughly rinsed with demineralized water, and sonicated for 5 minutes in demineralized water before use. The soft lenses were always new and were sterilized by dipping five times in NaCl before use. For AFM, the bottom of a lens case was cut out, as otherwise the shape of the case impeded the measurements. All CLs, with their convex sides up, as well as the bottoms of the storage cases, were fixed to a glass plate with double-sided sticky tape. Samples were surrounded by a ring of wax to keep a drop of 0.9% NaCl between the sample and the AFM cantilever.

Corneas

Porcine eyes were acquired from pigs (Kroon BV, Groningen, The Netherlands) recently killed for commercial purposes, not for use in the study. The eyes were transported to the laboratory within 1 hour after death and, on arrival, were rinsed for 5 minutes with 200 mL demineralized water, washed for 5 minutes in sterile 0.9% NaCl supplemented with penicillin (200 U/mL) and streptomycin (200 μg/mL), and then thoroughly washed five times in 200 mL sterile 0.9% NaCl on a shaking table, to reduce the amount of bacteria on the cornea to an undetectable level, when examined with bacteria counting plates (Petrifilm Aerobic Count Plates; 3M Microbiology, St. Paul, MN).

Corneas were dissected from the intact eyes with a sterile knife immediately after rinsing, fixed on wax with nails to prevent wrinkling, and then fixed to a glass plate for AFM, similar to the lens case bottoms and the CLs. AFM measurements on corneas thus prepared started within 2 hours after death and were made on their convex sides.

AFM Adhesion-Force Measurements

Bacteria from suspension were immobilized on tipless V-shaped cantilevers (DNP-0; VEECO, Woodbury, NY) by means of electrostatic interaction. The end of a cantilever was immersed in a droplet of 0.01% (wt/vol) poly-L-lysine solution (Sigma-Aldrich, Poole, UK) for 1 minute. After air drying for 2 minutes, it was dipped in a bacterial suspension for 1 minute to allow bacterial attachment. Bacterial probes were always freshly made for each experiment.

AFM measurements were performed at room temperature in 0.9% NaCl using a (Dimension 3100 system, Nanoscope IV; Digital Instruments, Santa Barbara, CA) with *z*-scan rates of 1.0 Hz, ramp sizes of 1.5 μm, and trigger threshold of 1 V. For each probe, force curves were measured with different surface–delay times (0, 10, 30, 60, and 90 seconds) on randomly chosen spots on the substratum surfaces, and repeated five times with one tip. Thirty force-distance curves, measured with six staphylococcal probes prepared from three separate cultures, were collected for each surface–delay time. To ensure that bacterial probes were not affected by a previous measurement, force-distance curves were made with 0 seconds contact time after each surface–delay time on a clean glass surface. If the adhesion-force with glass differed more than 1 nN from the initially measured value, the latest measurement was discarded and a new probe prepared.

Calibration of bacterial probes was performed with the thermal-tuning method, yielding spring constants of 0.054 (± 0.008) N/m. Subsequently, for each probe, the maximum adhesion forces F occurring in the retract force-distance curves were plotted as a function of the surface–delay time t according to

$$F = F_0 + (F_\infty - F_0)\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] \quad (1)$$

with F_0 being the maximum adhesion force at 0 seconds of contact time, F_∞ the maximum adhesion force after bond-strengthening, and τ the characteristic time needed for the adhesion force to strengthen.

Weibull Analysis of Adhesion Forces and Calculation of Transmission Probabilities

Staphylococcal transmission probabilities between the different surfaces, including the corneal surface, were calculated from Weibull analyses.[19] As a first step, all adhesion forces N in a given data set were ranked in ascending order to calculate the probability P_F of a force value F to occur according to

$$P_F = \frac{n}{N+1} \quad (2)$$

in which n is the rank number. Then, P_F is fitted to the Weibull equation

$$P_F = 1 - \exp\left[-\left(\frac{F - F_u}{F_n}\right)^m\right] \quad (3)$$

in which the constant F_u is the lowest level of force at which P_F approaches 0. In macroscopic bond-strength analyses, it is mostly assumed that F_u is 0, but this is not necessarily true in AFM force measurements.[18] The constant F_n is a difficult parameter to visualize and is generally referred to as a normalizing parameter. The constant m is the dependability of the bond (Weibull modulus). A high value of m indicates a close grouping of measured forces and high reliability of the data set, while a low value indicates a wide, long-tailed distribution.

Calculations of transmission probabilities were made on the basis of the Weibull distribution calculated for each combination of bacteria, lens case, CL type, or corneal surface. First, the median force between *S. aureus* 835 and a CL was determined, after which the Weibull distribution for the adherence of the bacterial strain to a lens case was used to calculate the transmission probability from case to CL ($T_{LC\text{-}CL}$),

under the influence of the median force exerted by the CL. This procedure is graphically illustrated in Figure 1. Next, the probability of transmission from CL to cornea ($T_{CL\text{-}C}$) was calculated by determining the probability of detachment of a bacterium from a CL under the influence of the median force between a bacterium and the cornea in the Weibull distribution of bacterial adhesion forces for the CL. The final transmission probability from lens case to cornea ($T_{LC\text{-}C}$) simply follows from $T_{LC\text{-}CL} \times T_{CL\text{-}C}$.

Bacterial Transmission Experiments

For transmission from case to CL, a sterilized lens case was first inoculated with 3 mL bacterial suspension for 30 minutes. After the case was removed from the bacterial suspension and rinsed once with 3 mL 0.9% NaCl, a CL was added and left to incubate for 90 seconds or 8 hours at room temperature in a closed lens case filled with 3 mL NaCl. After incubation, the CL and NaCl were removed from the case, and the number of adhering bacteria on the lens case surface and convex side of the CL were determined by contacting the CL with bacterial count plates (Petrifilm AC; 3M).[20–22] The plates were incubated in aerobic conditions at 37°C for 48 hours before the number of colonies appearing as red dots on the gel was recorded. Bacterial transmission from lens case to CL was calculated by

$$Transmission(\%) = \frac{n_{CL}}{n_{CL} + n_{LC}} \times 100\% \quad (4)$$

in which n_{CL} and n_{LC} are the number of bacteria adhering per square centimeter on the CL and lens case, respectively.

For transmission from CL to the cornea, a CL was placed with its convex side up in 5 mL staphylococcal suspension and incubated for 30 minutes with slight agitation on a rotating table. CLs were then rinsed five times in sterile 0.9% NaCl and put on porcine eyes, wetted with 50 μL of 0.9% NaCl supplemented with 2% TSB, incubated in 100% humidity for 90 seconds or 8 hours. After removal of the CLs from the eyes, the number of bacteria adhering to the concave side of the CL and on the porcine corneas was determined with the bacterial count plates, and the percentage transmission of bacteria from the CL to the cornea was calculated.

All transmission experiments were performed in triplicate with separately cultured bacteria.

Statistical Analysis

The adhesion forces were not normally distributed and are presented as the median and interquartile range. Differences between adhesion forces were analyzed using the nonparametric Kruskal-Wallis test, followed by Dunn's multiple-comparison post hoc test, when overall differences were significant at $P < 0.05$. Comparison of transmission probabilities from Weibull-analyses and transmission experiments were performed with Student's *t*-test (all analyses: SPSS ver. 16.0 for Windows; SPSS, Chicago, IL).

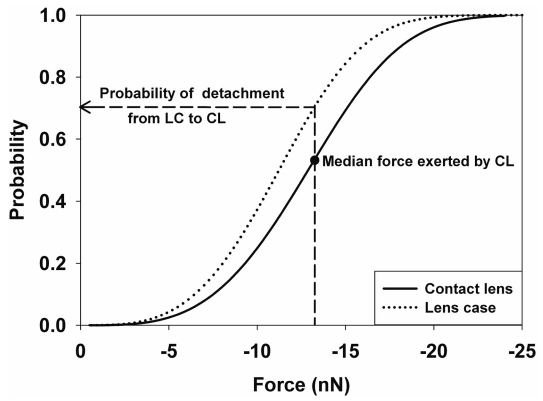

FIGURE 1. The calculation of transmission probabilities on the basis of Weibull distributions from a lens case to a CL surface. The median force exerted by a CL on a bacterium is projected to the Weibull distribution for the adhesion forces between lens case and bacterium and the probability of that force causing detachment of a bacterium from the lens case (LC) to the CL surface can be directly read.

RESULTS

Force–Distance Curves and Adhesion Forces

Figure 2 shows an example of the retraction force–distance curves for *S. aureus* 835 for a lens case surface, soft and rigid CLs, and a porcine cornea. The maximum adhesion force increased strongly with increasing surface–delay times and multiple minor adhesion peaks were observed to develop over time. The rigid CL exerted a stronger adhesion force than the soft CL, lens case, and corneal surface, especially after 90 seconds surface delay (Fig. 3). The corneal adhesion forces extend over the longest distance, presumably due to the stretching of adsorbed macromolecular components on the cornea.

Bond-strengthening as a function of the surface–delay time is shown in Figure 3. Median adhesion forces from 30 force–distance curves significantly strengthened within the first 10 seconds of contact and reached stable values within 30 to 60 seconds. Initial (F_0) and final (F_∞) adhesion forces, together with a characteristic time constant for the bond-strengthening process τ can be derived from a graph such as that shown in Figure 3 by using equation 1 and are summarized in Table 1. Staphylococcal adhesion forces all became stronger over time and bond-strengthening by a factor of 5 generally occurred within 20 to 30 seconds. Adhesion forces were significantly ($P < 0.05$) weaker on soft than on rigid CLs, cases, and corneas, before and after bond-strengthening. Median adhesion forces between staphylococci and lens cases were similar to the adhesion forces between staphylococci and cornea ($P > 0.05$).

Probability of Bacterial Transmission on the Basis of Weibull Analysis

Figure 4 shows the Weibull distributions for lens cases and soft and rigid CLs as well as for corneas, based on the adhesion forces and their spread, measured after a surface–delay of 90 seconds. Weibull moduli *m* for the distributions were relatively low, indicative of the large spread in data, but the data fitted the Weibull equation well (see data in Table 1). After the median adhesion force exerted by the CLs on staphylococci adhering to the case (Table 1) is calculated, these Weibull distributions can be used to find the transmission probability from case to CL and CL to cornea (Table 2). Transmission probabilities are thus not only determined by the magnitude of adhesion forces, but moreover by the shape of the Weibull distribution. Accordingly, staphylococcal transmission probabilities from a lens case to a rigid CL are predicted to be higher than to a soft CL. Inversely, transmission from a soft CL to the cornea is higher than that from a rigid CL according to the Weibull probabilities. The final transmission probability of *S. aureus* 835 from case to cornea with a soft CL as the intermediary is predicted to be slightly smaller (19%, see Table 2) than with a rigid CL as the intermediary (24%).

Bacterial Transmission Based on Experimental Results

The initial number of adhering staphylococci on contact lens cases before transmission to the CLs amounted 130 bacteria cm^{-2}, whereas on soft and rigid CLs before transmission to the corneas, the counts were 750 and 450 bacteria cm^{-2}, respectively (Table 2, footnote). The number of bacteria present on

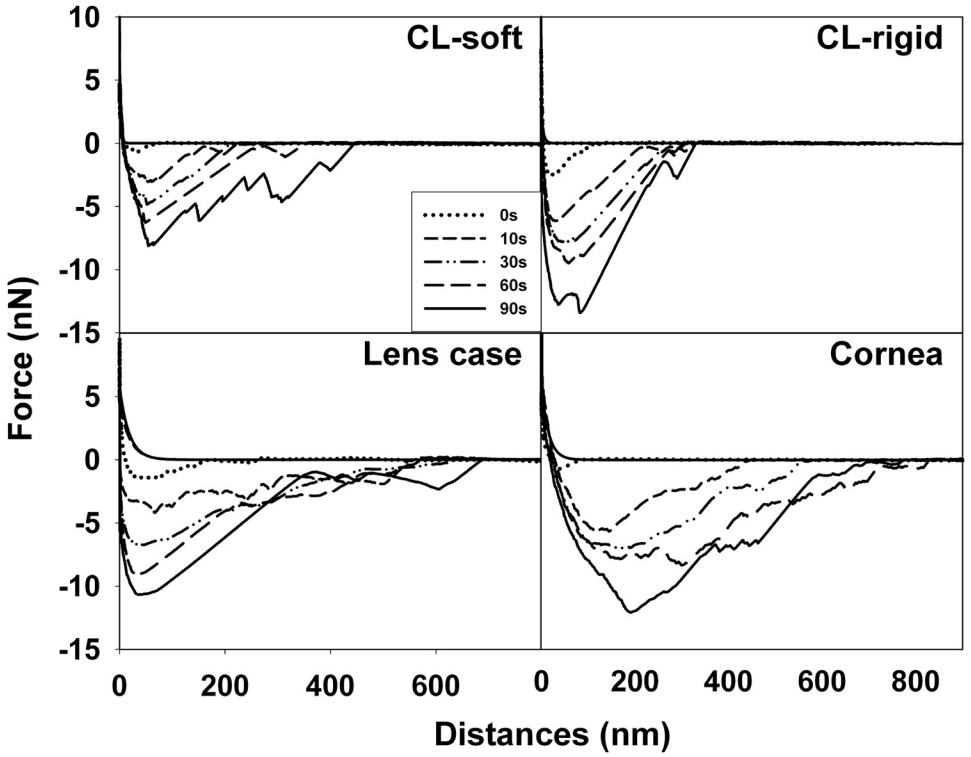

FIGURE 2. Examples of the retraction force–distance curves between *S. aureus* 835 attached to the AFM cantilever and a lens case surface, soft and rigid CLs, and porcine corneas after different surface–delay times.

the receiving surfaces were subsequently used to calculate the experimental transmission of staphylococci from lens case to CL ($T_{LC\text{-}CL}$) and from CL to cornea ($T_{CL\text{-}C}$) after different contact times (90 seconds and 8 hours), as also summarized in Table 2. Transmission of staphylococci from lens case to CL increased significantly with increasing contact time, but transmission from CL to cornea appeared to be independent of contact time, regardless of the CL type involved. Generally, rigid CLs attracted significantly more staphylococci from a case than did soft CLs ($P < 0.05$), whereas corneas attracted significantly more bacteria from soft than from rigid CLs ($P < 0.05$). There were no significant differences in final transmission of bacteria to corneas mediated by soft and rigid CLs, whereas the transmission probabilities derived from Weibull distributions fell within the range of data observed for the 90-second and 8-hour contact times.

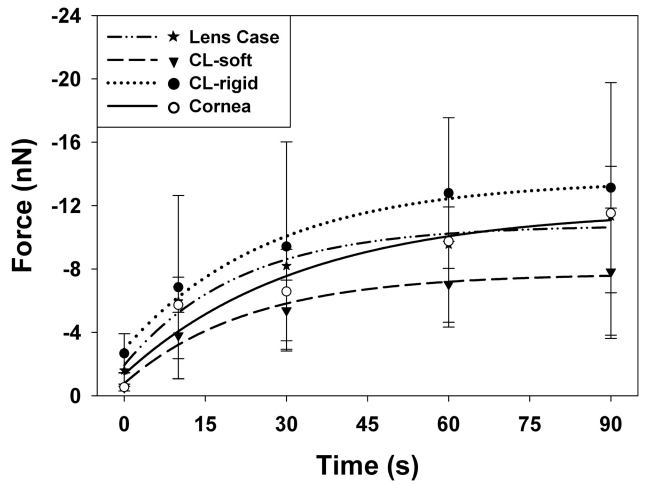

FIGURE 3. The maximum adhesion forces between an AFM cantilever with attached *S. aureus* 835 as a function of the surface–delay time with a lens case surface, soft and rigid CLs, and porcine corneas. Data are expressed as the median with interquartile ranges. The adhesion forces on soft CLs are significantly ($P < 0.05$) weaker than those on other surfaces, whereas adhesion forces on rigid CLs are significantly ($P < 0.05$) stronger than those on other surfaces.

DISCUSSION

In this study, we compared the transmission probabilities of *S. aureus* 835 from lens cases with corneas with soft and rigid CLs as an intermediary based on AFM adhesion-force measurements and Weibull analyses. Rigid CLs were predicted to have a slightly higher transmission probability than soft CLs, but the difference was not statistically significant. More important, Weibull analyses offered insight into the weakest link in the chain of events that lead to bacterial transmission and adhesion to the cornea. Prevention of bacterial transmission from case to

TABLE 1. Initial (F_0) and Final (F_∞) Adhesion Forces, Together with the Characteristic Time Constant τ for Bond-Strengthening, as Measured during Retraction of a *Staphylococcus*-Coated AFM Cantilever from a Lens Case and Soft and Rigid CLs and Porcine Corneas

	F_0 (nN)	F_∞ (nN)	τ (s)	m	R^2
Lens case	−1.9 ± 0.9	−10.8 ± 2.0	21 ± 8	2.85	0.97
Soft CL	−0.8 ± 0.6*	−7.7 ± 1.3*	23 ± 7	3.48	0.97
Rigid CL	−2.9 ± 0.7†	−13.6 ± 1.7†	27 ± 7	3.50	0.94
Cornea	−1.4 ± 1.4	−11.8 ± 3.9	33 ± 11	3.41	0.93

Data represent the median ± SE over 30 force-distance curves. The reliability of the data sets is further indicated by their Weibull moduli m. R^2 denotes goodness of fit of the adhesion force data to the Weibull equation.

* F_0 and F_∞ were the lowest on soft CLs, when compared with the other materials ($P < 0.05$).

† F_0 and F_∞ were the strongest on rigid CLs, when compared with the other materials ($P < 0.05$).

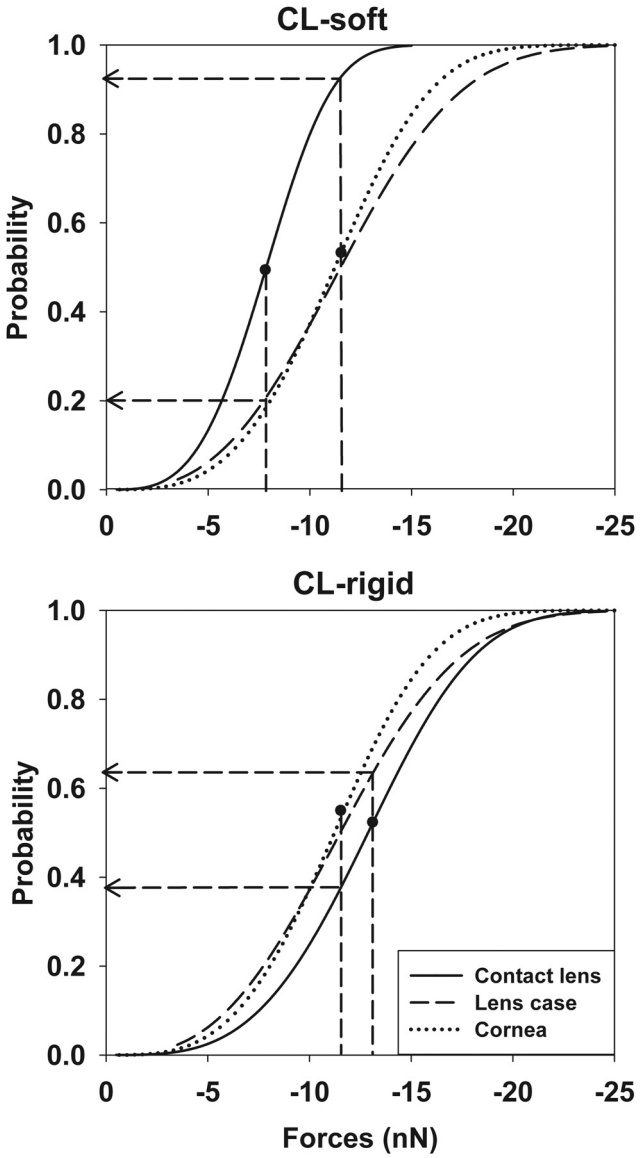

FIGURE 4. Probability of *S. aureus* 835 transmission from lens case to CLs and from soft and rigid CLs to corneas, according to the Weibull equation. (●) The median forces on the different Weibull curves of the donating surfaces; *dotted lines*: extrapolation to the Weibull curves of the receiving surfaces, from which the detachment probabilities can be directly read (see also Fig. 1).

cornea through a CL constitutes an intricate comparison of adhesion forces. A CL that strongly attracts bacteria will become more easily contaminated in the lens case, but will donate fewer bacteria to the cornea. Similarly, a case exerting strong adhesion forces on bacteria is favorable, because it is less likely to donate bacteria to a CL surface. Accordingly, for the current combination of cases and CLs, the soft CL was less likely to be contaminated by the lens case, but would donate bacteria more readily to the cornea. Admittedly, only one bacterial strain was used in this study, but also other bacterial strains, either in the planktonic phase (motile or nonmotile) or in a biofilm, would adhere to the surface, exerting the strongest attractive forces, which does not necessarily need to be the same surface for different bacterial strains or species.

Measurement of staphylococcal adhesion forces involved in transmission from one surface to another, included bond-strengthening between *S. aureus* 835 and CLs, lens cases, and corneas. The bond-strengthening observed over the first 20 to 30 seconds after initial contact with a surface is purely physicochemical in nature and not due to growth or excretion of extracellular polymeric substances, as it has also been observed for inert micrometer-sized particles.[23] Progressive development of hydrogen bonds over time after structural or protein rearrangements has been suggested as the main cause of bacterial bond-strengthening. *S. aureus* 835 is a hydrophilic strain that can interact with surfaces through hydrogen-bonding and in fact the minor peaks appearing in the flanks of the retracted AFM force–distance curves are indicative of hydrogen-bonding.[24] Importantly, these minor peaks are more prominent after a surface–delay than on initial contact. Although Weibull distributions for lens cases, CLs, and corneas could have been calculated on the basis of adhesion forces after different surface–delay times, we decided to only use the 90-second surface–delay data, as they correspond with the contact time applied in actual bacterial transmission and because they represent a plateau level of bond-strengthening. Within the 8 hours of contact applied in transmission experiments, strengthening processes other than that included in initial bond-strengthening may occur. Unfortunately, the AFM equipment does not allow the use of surface–delay times of 8 hours.

Weibull analysis is often used to calculate the probability of failure of macroscopic adhesive bonds,[25] but can be used equally well for AFM adhesion forces. The final probability of transmission from contaminated lens cases to corneas predicted by the Weibull analysis coincides well with results from actual staphylococcal transmission experiments, and the transmissions from case to the intermediary CL and from CL to cornea are clearly predictable for both soft and rigid CLs. This predictability is particularly true of surface delay and contact times of 90 seconds, which suggest that further increasing contact times to 8 hours may reveal additional effects on the adhesion forces, not accounted for in bond-strength measurements over only 90 seconds.

Summarizing, we present a novel adhesion-force–based method of determining the transmission of bacteria from lens case surfaces to corneas. The proposed method not only corresponds well with actual staphylococcal transmission studies, but moreover enables analysis of transmissions to intermediaries on the basis of force analysis and can be easily extended to include, for instance, different temperatures during AFM force measurements.

TABLE 2. Actual Bacterial Transmissions and Predictions According to Weibull Distributions Calculated for Adhesion Forces Measured after a 90-second Surface-Delay Time

Lens Type/Method	$T_{LC\text{-}CL}$	$T_{CL\text{-}C}$	$T_{LC\text{-}CL\text{-}C}$
Soft CL			
90-s transmission	25 ± 3*	65 ± 7	16 ± 10
8-h transmission	49 ± 3*	66 ± 11	33 ± 4
Weibull prediction	21	93	19†
Rigid CL			
90-s transmission	54 ± 3*	38 ± 6	21 ± 9
8-h transmission	68 ± 5*	42 ± 3	29 ± 8
Weibull prediction	63	37	24†

Actual transmissions are presented as the average percentages ± SE over three measurements of separate bacterial cultures. The average number of staphylococci on the lens case and the soft and rigid CLs before transmission amounted to 130, 750, and 450 bacteria cm^{-2}, respectively.

* Significant difference between $T_{LC\text{-}CL}$ and $T_{CL\text{-}C}$ ($P < 0.05$).

† Transmission probabilities from lens case to cornea ($T_{LC\text{-}CL\text{-}C}$), derived from Weibull distributions, fall within the range of data observed for 90-second and 8-hour contact times.

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